Speciation of *Campylobacter coli*, *C. jejuni*, *C. helveticus*, *C. lari*, *C. sputorum*, and *C. upsaliensis* by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry†

Robert E. Mandrell,* Leslie A. Harden, Anna Bates, William G. Miller,
William F. Haddon, and Clifton K. Fagerquist

*Produce Safety and Microbiology Research Unit, USDA Agricultural Research Service, Western Regional
Research Center, 800 Buchanan St., Albany, California 94710*

Multiple strains of *Campylobacter coli*, *C. jejuni*, *C. helveticus*, *C. lari*, *C. sputorum*, and *C. upsaliensis* isolated from animal, clinical, or food samples have been analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Whole bacterial cells were harvested from colonies or confluent growth on agar and transferred directly into solvent and then to a spot of dried 3-methoxy-4-hydroxycinnamic acid (matrix). Multiple ions in the 5,000- to 15,000-Da mass range were evident in spectra for each strain; one or two ions in the 9,500- to 11,000-Da range were consistently high intensity. "Species-identifying" biomarker ions (SIBIs) were evident from analyses of multiple reference strains for each of the six species, including the genome strains *C. jejuni* NCTC 11168 and *C. jejuni* RM1221. Strains grown on nine different combinations of media and atmospheres yielded SIBI masses within ±5 Da with external instrument calibration. The highest-intensity *C. jejuni* SIBIs were cytosolic proteins, including GroES, HU/HCj, and RplL. Multiple intraspecies SIBIs, corresponding probably to nonsynonymous nucleotide polymorphisms, also provided some intraspecies strain differentiation. MALDI-TOF MS analysis of 75 additional *Campylobacter* strains isolated from humans, poultry, swine, dogs, and cats revealed (i) associations of SIBI type with source, (ii) strains previously speciated incorrectly, and (iii) "strains" composed of more than one species. MALDI-TOF MS provides an accurate, sensitive, and rapid method for identification of multiple *Campylobacter* species relevant to public health and food safety.

Thermophilic strains of *Campylobacter* account for a high percentage of the estimated 76 million yearly incidents of food-borne illness in the United States (49). Although many outbreaks of *Campylobacter* disease have been reported in the last 25 years, most illnesses occur sporadically and are caused by *C. jejuni* (51). Of the 16 species of the genus *Campylobacter* identified to date, at least eight have been identified as potential human gastrointestinal pathogens: *C. jejuni*, *C. coli*, *C. lari*, *C. fetus*, *C. upsaliensis*, *C. sputorum*, *C. concisus*, and *C. curvus* (1, 7, 15, 22, 23, 29, 37, 38, 47, 48, 53, 60, 62, 64, 66). Approximately 0.1% of *C. jejuni* cases are associated with a serious paralytic disease, Guillain-Barré syndrome (55). Also, *C. rectus* and possibly *C. showae* are associated with periodontal disease (44).

There are a variety of existing assays for confirming *Campylobacter* by genus and species. These include biochemical (2), genetic (PCR) (3, 6, 20, 32, 71), immunochemical (34, 36, 45), chemotaxonomic fatty acid profiling (9), protein one-dimensional gel electrophoresis methods (67) and, recently, a microarray-based method (69). Some of the limitations in these methods are hippuricase-negative

C. jejuni strains (24, 30, 43), the necessity for multiple hybridization steps and/or primers with multispecies PCR-based assays (3, 6, 20, 25, 31, 43, 59, 68, 71), specificity issues with 16S rRNA PCR (42, 43; unpublished observations), and chemotaxonomic methods (9, 63), and only limited information (e.g., species) is obtained. Details of the fine specificities of immunochemical reagents and assay methods and comparisons of results with multiple strains of *C. jejuni*, *C. coli*, and emerging *Campylobacter* species have been limited (34, 36, 56).

Mass spectrometric methods for analyzing whole bacterial cells for intact proteins, including the identification of protein biomarker ions, have been reported previously (5, 11, 16, 28, 35, 70). In a preliminary study most relevant to this work, *Campylobacter* and *Helicobacter* strains were analyzed by matrix-assisted laser desorption–time of flight mass spectrometry (MALDI-TOF MS), and biomarker ions in the 10- to 20-kDa range (presumably proteins) were reported to be the most discriminatory of those observed (73).

The major advantages of MALDI-TOF MS for analyzing bacteria are (i) ease and speed of analysis, (ii) identification of mixed cultures, and (iii) rapid identification of candidate biomarkers, even when minimal genetic data are available. We have developed an expanded MALDI-TOF MS method for analyzing multiple *Campylobacter* species and applied this method to determine species, and limited subspecies classes, of

suspected *Campylobacter* strains isolated from a variety of food and animal sources.

MATERIALS AND METHODS

Bacterial strains. The RM strain number is the designation for strains in the Produce Safety and Microbiology Research Unit strain file. Sources and other information about the strains are noted in Table 1.

Bacterial isolation and culture conditions. *C. coli* and *C. jejuni* strains were maintained on brucella agar (BA) containing, per liter, 28 g brucella broth (BD Biosciences, San Jose, CA), supplements (0.25 g $FeSO_4$, 0.25 g sodium meta-bisulfite [anhydrous], 0.25 g sodium pyruvate [anhydrous]) and 15 g of Bacto agar (BD Biosciences) as described previously (2, 50). Other *Campylobacter* strains were grown on BA and/or brain heart infusion agar medium containing 37 g of brain heart infusion broth medium, 6 g yeast extract, 15 g Bacto agar (BD Biosciences), and 100 ml laked horse blood (Hema Resource and Supply, Aurora, OR) per liter. All *Campylobacter* strains were grown under microaerophilic conditions at 37°C or 42°C by placing plates in a sealable plastic bag with a gas mixture of 10% CO_2, 5% H_2, and 85% N_2 (microaerophilic gas) or a commercial sachet (CampyPak; Oxoid, Inc.). In one experiment to compare the effects of different growth conditions on species-identifying biomarker ions (SIBIs), *C. jejuni* strain RM1221 was grown also on BA with 5% laked horse blood (BAB) and GC agar medium, which contains GC medium base and 1% Bacto agar, and supplemented with final concentrations of 0.3% glucose, 0.00084% ferric nitrate (nonahydrate), 0.01% L-glutamine, 0.001% thiamine pyrophosphate, and 0.005% L-cysteine, as described previously (46, 72). The media were incubated in a microaerophilic atmosphere as above or in 5% CO_2, 5% O_2, 90% N_2 at either 37°C or 42°C.

Some *C. upsaliensis* and *C. helveticus* strains analyzed in this study were isolated by a membrane filtration method modified from one described previously (41). An approximately 20% suspension of cat or dog feces in saline was incubated at 37°C for 30 min, and then four 50-μl samples were applied at different locations on a 0.6-μm membrane filter (ME26; 47 mm; Schleicher & Schuell) placed on top of a BAB plate. The sample was incubated on the filter at room temperature for 15 min, and then any solution remaining was removed and the filter was removed with sterile tweezers. The plate was placed in a plastic sealable bag (Ziploc Freezer; Johnson and Son), and the bag was filled with 10% H_2, 10% CO_2, and 80% N_2 and evacuated at least twice before final filling with gas; the bag and plate were incubated at 37°C for 48 to 72 h. Suspect *Campylobacter* colonies were subcultured on BAB for further analysis. The majority of the colonies were *Campylobacter* species.

Materials. Acetonitrile, ferrous sulfate, sodium metabisulfite, sodium pyruvate, brucella agar, and Bacto agar were purchased from Fisher Scientific (Pittsburgh, PA). Trifluoroacetic acid (TFA) and 3-methoxy-4-hydroxycinnamic acid (ferulic acid) were purchased from Aldrich Chemical (Milwaukee, WI). Adrenocorticotropic hormone clip (18-39), angiotensin II, substance P, bovine insulin, and horse heart myoglobin were purchased from Sigma Chemical Company (St. Louis, MO). Purified water (Milli-Q system; Millipore, Bedford, MA) was used for all experiments.

Preparation of bacterial cell lysates and MALDI sample targets. Cells were transferred from the plate to the extraction tube with a 1.0-μl disposable inoculating loop (NUNC Brand Products, Denmark); this volume corresponded to approximately 10^9 cells. The extraction tube was a 2.0-ml conical screw cap microtube (QSP; catalog no. 522; Porex Bio Products Inc.) with cap and O-ring. The extraction solution contained 0.5 ml of 33% HPLC-grade acetonitrile (Fisher Scientific, Fair Lawn, NJ), 67% HPLC-grade water (Burdick & Jackson, Muskegon, MI), 0.1% sequencing-grade TFA (Sigma-Aldrich, St. Louis, MO), and ~40 mg of 0.1-mm zirconia-silica beads (catalog no. 11079101z; BioSpec Products Inc., Bartlesville, OK). Extraction tubes were then tightly capped, placed into a 96-well aluminum holder, and agitated for 60 s on a beadbeater (Mini-beadbeater-96+; BioSpec Products, Bartlesville, OK). Bead beating for longer than 60 s was avoided to minimize sample heating due to bead friction. The solution was frothy and opaque after this step. Tubes were then centrifuged for 4 to 5 min at 10,600 × *g* (model 4515C; Eppendorf, Hamburg, Germany). The undissolved cellular material and beads at the bottom of the tube were pelleted by centrifugation, after which the supernatant was clear (bacterial cell lysate).

A saturated solution of *trans*-4-hydroxy-3-methoxy-cinammic acid (Sigma-Aldrich, St. Louis, MO) also known as ferulic acid, was prepared in 0.5 ml of 33% acetonitrile, 67% water, and 0.1% TFA (saturated matrix solution). A working matrix solution was prepared by adding 100 μl of the saturated matrix solution to 200 μl of 33% acetonitrile, 67% water, and 0.1% TFA. The solution was then briefly vortexed, 0.5 μl was deposited on a sample square of a 7 by 7 spot homemade stainless steel MALDI target plate, and the matrix solution was allowed to air dry at room temperature. Finally, 0.5 μl of bacterial cell lysate-supernatant was deposited onto the dried matrix and allowed to dry. This was repeated for each bacterial lysate sample.

Mass spectrometry. All experiments were run in the positive ion mode on a Bruker Daltonics (Billerica, MA) Reflex II instrument using the reflectron mode with delayed extraction; the delayed extraction setting was set to "medium." Ionization of molecules in the samples was achieved with a nitrogen laser (337 nm). A minimum sum of 200 laser shots per sample was used to generate each ion spectrum used for analysis. The instrument was calibrated externally before each batch run of test samples using the adrenocorticotropic hormone clip (peptide 18-39), angiotensin II, substance P, bovine insulin, and horse heart myoglobin as standards.

Identification of SIBI proteins based on mass determined by MALDI-TOF MS. The masses of SIBIs determined by MALDI-TOF MS were compared to the predicted molecular weights of proteins encoded by the annotated genes of *C. jejuni* strain NCTC 11168 (58) and *C. jejuni* strain RM1221 (65) for a tentative protein identification. The *C. jejuni* SIBI type 1 was confirmed by tryptic mass mapping (26). A tentative identification of other SIBIs was determined similarly from the partial genome sequences of *C. coli* strain RM2228, *C. lari* strain RM2100, and *C. upsaliensis* strain RM3195 (21).

MAbs. Two monoclonal antibodies (MAbs) shown previously to be specific for *C. jejuni* (anti-*Cj*) or both *C. jejuni* and *C. coli* (anti-*Cc/Cj*) were used in some experiments in a whole-cell enzyme-linked immunosorbent assay to identify *C. coli* and *C. jejuni* strains. Strains were cultured on BA or BAB, and the bacteria were harvested and then suspended in phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) to an optical density at 620 nm of 0.2 to 0.3. Seventy microliters of the suspension was added to polystyrene assay wells (Immulon II [Dynatech, Chantilly, Va.] or Maxisorp [Nunc, Roskilde, Denmark]). The plates were allowed to dry by incubating them for 18 to 24 h at 37°C, and they were washed three times with Tris-HCl-buffered saline containing 1% Tween 20 (TBS-Tween) or phosphate-buffered saline–Tween (diluents) and then with a final rinse of pyrogen-free water. Nonspecific binding sites on the wells were blocked by adding 200 μl of 1% casein blocking buffer and then incubating the plate at room temperature for 1 h. The plates were rinsed and washed as described above and used immediately, or they were stored in a desiccator at 4°C for later use. Dilutions of MAbs (100 μl) diluted in 1% bovine serum albumin in TBS-Tween were added to wells, and the wells were incubated with gentle shaking for 1 to 2 h at room temperature. Wells were emptied and then washed and rinsed with water. Bound MAb was detected by adding 100 μl (0.2 μg) of alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (Zymed, South San Francisco, CA), incubating the wells for 1 h with shaking, and then washing and rinsing the wells with water. One hundred microliters of *p*-nitrophenylphosphate (1 mM in 10 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.5) was added, and the absorbance at 405 nm was determined on a Spectramax 340 microplate reader (Molecular Devices, Sunnyvale, CA) (45).

Hippuricase activity. Hippuricase activity was determined by a method described previously (24).

MLST. Selected strains were analyzed by a multilocus sequence typing (MLST) method as described previously (52).

Fluorescent *Campylobacter* strains. *C. jejuni* strain RM1221 carrying a plasmid encoding cyan fluorescent protein (CFP) has been described previously (50). *C. lari* strain RM2100 was transformed with the same plasmid encoding green fluorescent protein (GFP) by a similar method (50).

RESULTS

MALDI-TOF MS spectra obtained for *Campylobacter* species reference strains. Initial MALDI-TOF MS studies were initiated with well-characterized reference *C. coli* and *C. jejuni* strains, and then the analysis was expanded to *C. lari*, *C. upsaliensis*, *C. helveticus*, and *C. sputorum* strains. In a typical analysis of *Campylobacter* strains by MALDI-TOF MS, 10 to 20 prominent ion peaks were noted in the spectra in the region between 6,000 and 15,000 Da, with the highest-intensity peaks consistently in the range of 9,000 to 10,300 Da. Figures 1 to 5, below, are representative generally of results obtained with multiple strains of each of the six species tested. Comparisons of spectra for multiple strains within a species revealed that multiple ions corresponded to a characteristic "fingerprint."

TABLE 1. Strains used in the study

PSM strain no.	Putative species	Source	Location	Additional strain info.[a]	Species by MALDI-TOF MS[b]
RM1048	C. jejuni	Human	Canada	ATCC 43432, HS4	C. jejuni
RM1050	C. jejuni	Human	Canada	ATCC 43449, HS23	C. jejuni
RM1051	C. coli	Human	Canada	ATCC 43479, HS30	C. coli
RM1155	C. jejuni	Human	Canada	HL1	C. jejuni
RM1156	C. jejuni	Human	Canada	HL2	C. jejuni
RM1158	C. jejuni	Human	Canada	HL5	C. jejuni
RM1159	C. jejuni	Human	Canada	HL6	C. jejuni
RM1161	C. coli	Human	Canada	HL8	C. coli
RM1163	C. jejuni	Human	Canada	HL11	C. jejuni
RM1164	C. jejuni	Human	Canada	HL15	C. jejuni
RM1165	C. jejuni	Chicken	Canada	HL17	C. jejuni
RM1166	C. coli	Chicken	Canada	HL21	C. coli
RM1167	C. jejuni	Human	Canada	HL28	C. jejuni
RM1168	C. jejuni	Human	Canada	HL36	C. jejuni
RM1169	C. coli	Human	Canada	HL55	C. coli
RM1199	C. jejuni	Chicken, processing water	United States	This study	C. jejuni
RM1216	C. jejuni	Chicken, retail	United States	This study	C. jejuni
RM1221	C. jejuni	Chicken, retail	United States	This study and reference 50	C. jejuni
RM1244	C. jejuni	Human	United States	CDHS, 90A2737	C. jejuni
RM1246	C. jejuni	Human	United States	CDHS, 92A3120	C. jejuni
RM1247	C. jejuni	Human	United States	CDHS, 96A11074	C. jejuni
RM1292	C. jejuni	Turkey	United States	This study	C. jejuni
RM1327	C. jejuni	Turkey	United States	This study	C. jejuni
RM1343	C. coli	Turkey	United States	This study	C. coli
RM1352	C. jejuni	Turkey	United States	This study	C. jejuni
RM1357	C. coli	Turkey	United States	This study	C. coli
RM1367	C. jejuni	Turkey	United States	This study	C. jejuni
RM1372	C. jejuni	Turkey	United States	This study	C. jejuni
RM1373	C. coli	Turkey	United States	This study	C. coli
RM1374	C. jejuni	Turkey	United States	This study	C. jejuni
RM1376	C. jejuni	Turkey	United States	This study	C. jejuni
RM1399	C. coli	Turkey	United States	This study	C. coli
RM1420	C. jejuni	Turkey	United States	This study	C. jejuni
RM1421	C. jejuni	Turkey	United States	This study	C. jejuni
RM1438	C. jejuni	Turkey	United States	This study	C. jejuni
RM1462	C. lari	Human	Not known	ATCC 35223	C. lari
RM1485	C. sputorum biovar fecalis	Ovine	Not known	ATCC 33709	C. sputorum bv. fecalis
RM1488	C. upsaliensis	Human	United States	ATCC 49815	C. upsaliensis
RM1495	C. sputorum biovar bulbulus	Not determined	Not known	ATCC 33491	C. sputorum bv. bulbulus
RM1862	C. jejuni	Human	United Kingdom	HL4, NCTC 11168 (58)	C. jejuni
RM1875	C. coli	Swine	Not known	ATCC 33559	C. coli
RM1876	C. coli	Human	Belgium	ATCC 43473, HS14	C. coli
RM1877	C. coli	Human	Canada	ATCC 43482, HS46	C. coli
RM1878	C. coli	Human	Canada	ATCC 43474, HS20	C. coli
RM1879	C. coli	Human	Canada	ATCC 43479, HS30	C. coli
RM1880	C. coli	Human	United States	ATCC 43485, HS49	C. coli
RM1881	C. jejuni	Human	United States	ATCC 33291	C. jejuni
RM1882	**C. jejuni[c]**	**Human**	**United States**	**ATCC 43429, HS1**	**C. coli + C. jejuni**
RM1883	C. jejuni	Calf	Canada	ATCC 43430, HS2	C. jejuni
RM1885	C. jejuni	Not known	Not known	ATCC 43463, HS44	C. jejuni
RM1886	C. jejuni	Bovine	Not known	ATCC 33560	C. jejuni
RM1887	C. lari	Human	United Kingdom	ATCC 35223, NCTC 11458	C. lari
RM1888	C. lari	Avian	Northern Europe	ATCC 35221, NCTC 11352	C. lari
RM1889	C. lari	Dog	Northern Europe	ATCC 35222, NCTC 11457	C. lari
RM1890	**C. lari**	**Human**	**Canada**	**ATCC 43675**	**C. lari (+ C. jejuni?)**
RM1891	C. coli	Chicken farm	United States	K20	C. coli
RM1892	C. jejuni	Chicken farm	United States	K21	C. jejuni
RM1893	C. jejuni	Chicken farm	United States	K22	C. jejuni
RM1894	C. jejuni	Chicken farm	United States	K23	C. jejuni
RM1895	C. jejuni	Chicken farm	United States	K24	C. jejuni
RM1896	C. coli	Swine farm	United States	1921	C. coli
RM1897	C. coli	Swine farm	United States	1926	C. coli
RM1898	C. coli	Swine farm	United States	1931	C. coli

TABLE 1—*Continued*

PSM strain no.	Putative species	Source	Location	Additional strain info.[a]	Species by MALDI-TOF MS[b]
RM1899	*C. coli*	Swine farm	United States	1936	*C. coli*
RM1900	*C. coli*	Swine farm	United States	1941	*C. coli*
RM1901	*C. coli*	Swine farm	United States	2151	*C. coli*
RM1902	*C. coli*	Swine farm	United States	2156	*C. coli*
RM1904	*C. coli*	Swine farm	United States	2171	*C. coli*
RM1905	*C. coli*	Swine farm	United States	2176	*C. coli*
RM1906	*C. coli*	Swine farm	United States	2181	*C. coli*
RM1907	*C. coli*	Swine farm	United States	2191	*C. coli*
RM1908	*C. coli*	Swine farm	United States	2196	*C. coli*
RM1909	*C. jejuni*	Human	Canada	ATCC 43431, TGH9011, HS3	*C. jejuni*
RM1910	*C. jejuni*	Chicken	United States	This study	*C. jejuni*
RM2089	***C. sputorum* bv. *fecalis***	**Ovine**	**Not known**	**CDC D1137**	***C. upsaliensis***
RM2090	*C. sputorum* bv. *fecalis*	Ovine	United States	CDC D1169, NCTC 11415	*C. sputorum* bv. *fecalis*
RM2091	*C. sputorum* bv. *fecalis*	Ovine	Not known	CDC D1138	*C. sputorum* bv. *fecalis*
RM2092	*C. upsaliensis*	Human	United States	CDC D1673	*C. upsaliensis*
RM2093	*C. upsaliensis*	Human	United States	CDC D1178	*C. upsaliensis*
RM2095	*C. jejuni* subsp. *doylei*	Human (blood)	United States	CDC 2722	*C. jejuni* subsp. *doylei*
RM2096	*C. jejuni* subsp. *doylei*	Human	United States	CDC D2781	*C. jejuni* subsp. *doylei*
RM2098	*C. lari*	Human	United States	CDC D70	*C. lari*
RM2099	*C. lari*	Human	United States	CDC D71	*C. lari*
RM2100	*C. lari*	Human	United States	CDC D67	*C. lari*
RM2225	***C. jejuni***	**Chicken**	**United States**	**MDR**	***C. coli***
RM2228	***C. jejuni***	**Chicken**	**United States**	**MDR**	***C. coli***
RM2230	*C. coli*	Chicken	United States	MDR	*C. coli*
RM2231	*C. coli*	Chicken	United States	MDR	*C. coli*
RM2236	*C. coli*	Chicken	United States	MDR	*C. coli*
RM2241	***C. jejuni***	**Chicken**	**United States**	**MDR**	***C. coli***
RM2243	***C. jejuni***	**Chicken**	**United States**	**MDR**	***C. coli***
RM3195	*C. upsaliensis*	Human	South Africa	300.94	*C. upsaliensis*
RM3228	*C. helveticus*	Feline	Switzerland	ATCC 51209, NCTC 12470	*C. helveticus*
RM3776	*C. upsaliensis*	Human	South Africa	365.96	*C. upsaliensis*
RM3777	*C. upsaliensis*	Human	South Africa	3.97	*C. upsaliensis*
RM3778	*C. upsaliensis*	Human	South Africa	5.97	*C. upsaliensis*
RM3807	***C. upsaliensis***	**Feline**	**United States**	**This study**	***C. helveticus***
RM3808	*C. upsaliensis*	Canine	United States	This study	*C. upsaliensis*
RM3809	*C. upsaliensis*	Canine	United States	This study	*C. upsaliensis*
RM3810	*C. upsaliensis*	Feline	United States	This study	*C. upsaliensis*
RM3812	*C. upsaliensis*	Canine	United States	This study	*C. upsaliensis*
RM3937	*C. upsaliensis*	Human	United States	CDHS-LA #3	*C. upsaliensis*
RM3939	*C. upsaliensis*	Human	United States	CDHS-LA #21	*C. upsaliensis*
RM3940	*C. upsaliensis*	Human	United States	CDHS-LA #54	*C. upsaliensis*
RM3941	*C. upsaliensis*	Human	United States	CDHS-LA #58	*C. upsaliensis*
RM3942	*C. upsaliensis*	Human	United States	CDHS-LA #675	*C. upsaliensis*
RM3943	*C. upsaliensis*	Human	United States	CDHS-LA #1419	*C. upsaliensis*
RM3945	*C. upsaliensis*	Canine	United States	CDHS-LA #3846	*C. upsaliensis*
RM3946	*C. upsaliensis*	Canine	United States	CDHS-LA #1883	*C. upsaliensis*
RM3947	*C. upsaliensis*	Canine	United States	CDHS-LA #1884	*C. upsaliensis*
RM3948	*C. upsaliensis*	Canine	United States	CDHS-LA #1885	*C. upsaliensis*
RM3949	*C. upsaliensis*	Canine	United States	CDHS-LA #2041	*C. upsaliensis*
RM3950	*C. upsaliensis*	Canine	United States	CDHS-LA #2042A	*C. upsaliensis*
RM4039	*C. upsaliensis*	Human	South Africa	SA 181.00	*C. upsaliensis*
RM4040	*C. upsaliensis*	Human	South Africa	101.01	*C. upsaliensis*
RM4042	*C. upsaliensis*	Human	South Africa	106.01	*C. upsaliensis*
RM4087	*C. helveticus*	Feline	Switzerland	CCUG 30566	*C. helveticus* + *C. upsaliensis*?[d]
RM4088	*C. helveticus*	Feline	Sweden	CCUG 34016	*C. helveticus*
RM4120	*C. sputorum* bv. *paraureolyticus*	Human	Canada	LMG11764	*C. sputorum* bv. *paraureolyticus*
RM4121	*C. sputorum* bv. *fecalis*	Ovine	United Kingdom	CCUG 20703	*C. sputorum* bv. *fecalis*

TABLE 1—*Continued*

PSM strain no.	Putative species	Source	Location	Additional strain info.[a]	Species by MALDI-TOF MS[b]
RM4124	*C. upsaliensis*	Canine	Switzerland	CCUG 19607	*C. upsaliensis*
RM4244	*C. upsaliensis*	Human	Belgium	LMG9104	*C. upsaliensis*
RM4245	*C. upsaliensis*	Human	Belgium	LMG9108	*C. upsaliensis*
RM4246	*C. upsaliensis*	Human	Belgium	LMG9114	*C. upsaliensis*
RM4248	*C. upsaliensis*	Human	Belgium	LMG9125	*C. upsaliensis*
RM4249	*C. upsaliensis*	Human	Belgium	LMG9129	*C. upsaliensis*
RM4250	*C. upsaliensis*	Human	Belgium	LMG9140	*C. upsaliensis*
RM4252	*C. upsaliensis*	Human	Belgium	LMG9226	*C. upsaliensis*
RM4254	*C. upsaliensis*	Human	Belgium	LMG9234	*C. upsaliensis*
RM4257	*C. upsaliensis*	Human	Belgium	LMG9265	*C. upsaliensis*
RM4258	*C. upsaliensis*	Human	Belgium	LMG9269	*C. upsaliensis*
RM4385	*C. upsaliensis*	Feline	United States	This study	*C. upsaliensis*
RM4386	*C. upsaliensis*	Feline	United States	This study	*C. upsaliensis*
RM4387	**C. upsaliensis**	**Feline**	**United States**	**This study**	**C. helveticus**
RM4388	**C. upsaliensis**	**Feline**	**United States**	**This study**	**C. helveticus**
RM4389	**C. upsaliensis**	**Feline**	**United States**	**This study**	**C. helveticus**
RM4390	**C. upsaliensis**	**Feline**	**United States**	**This study**	**C. helveticus**
RM4391	**C. upsaliensis**	**Feline**	**United States**	**This study**	**C. helveticus**
RM4392	**C. upsaliensis**	**Feline**	**United States**	**This study**	**C. helveticus**

[a] The original strain numbers are noted also, if known. MDR, multidrug resistant; HS, Penner heat-stable serotype; HL, Lior heat-sensitive (labile) serotype; CDC, Centers for Disease Control and Prevention; ATCC, American Type Culture Collection, United States; NCTC, National Type Culture Collection, United Kingdom; CDHS-LA, California Dept. of Health Services—LA Laboratory; CCUG, Culture Collection of the University of Göteborg, Sweden; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Belgium.
[b] Species determined by MALDI-TOF MS (Table 2; see also Table S1 in the supplemental material).
[c] Boldface type indicates a "strain" with results inconsistent with original species designation.
[d] MLST data indicate possible mixture of *C. helveticus* and *C. upsaliensis* or *C. helveticus*-*C. upsaliensis* chimeric alleles.

Samples analyzed at different times over the course of several hours after calibration yielded results that were reproducible to 0.03% (data not shown), indicating that the precision of the method was sufficient for rapid determination of *Campylobacter* species. Mass abundance was reflected by the height of a peak; however, peak height was not useful generally for species identification or accurate quantification of cells. Masses illustrated in Fig. 1 to 5, below, for *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. helveticus* and *C. sputorum*, respectively, are the values determined for an individual experiment. The molecular weights of putative SIBIs were determined by averaging the results for multiple strains tested in multiple experiments. The precision of the externally calibrated data was approximately ±10 Da.

Designation of SIBIs from MALDI-TOF MS analysis of reference strains. Ions detected for *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. helveticus*, and *C. sputorum* biovar *fecalis* reference strains by MALDI-TOF MS (see Fig. 1 to 5, below) were designated as a candidate initially if they occurred in an approximate relative abundance of >10% of the most abundant ion in an experiment, were at least 10 Da different from other SIBIs, and were relatively conserved for a species. For example, *C. jejuni* strain RM1862 (NCTC 11168) (Fig. 1A) had prominent ions at 10,275 and 13,728, whereas neither ion was present in spectra for *C. coli* strains RM1161 and RM1051 (Fig. 2A and B). It was observed that some ions appeared common between species, e.g., 7,035- (7,036-) and 8,153- (8,154-) Da ions occur in both *C. coli* and *C. jejuni* strains (Fig. 1 and 2), but not in *C. lari* and *C. upsaliensis* strains (Fig. 3 and 4). For *C. lari* and *C. upsaliensis* strains, minor-intensity ions nearest to the 7,035- (7,036-) and 8,153- (8,154-) Da ions occurred at ~6,998 (*C. lari*) and 7,063 (*C. upsaliensis*) Da and at ~8,000 Da (*C. lari* and *C. upsaliensis*), respectively.

MALDI-TOF analysis of *C. coli*, *C. jejuni*, and two strains of *C. jejuni* subsp. *doylei*. Analysis of multiple reference strains of the same species, however, occasionally yielded spectra with a SIBI that varied slightly in mass from that identified initially. For example, a major peak was observed at ~10,275 Da (designated SIBI type 1) in profiles for most *C. jejuni* strains (Fig. 1A), but a 10,307-Da ion (designated SIBI type 2) was the nearest mass ion observed for other strains (Fig. 1B).

FIG. 1. MALDI-TOF MS spectra of whole-cell extracts of *C. jejuni* reference strains. Spectra representative of MS profile type 1 and profile type 2 were obtained from *C. jejuni* strains RM1862 (NCTC 11168) (A) and RM1438 (B), respectively. The relative intensities of the ions are shown on the *y* axis, and the masses (Da) of the ions are shown on the *x* axis.

FIG. 2. MALDI-TOF MS spectra of whole-cell extracts of *C. coli* reference strains. Spectra representative of MS profile type 1 and profile type 2 were obtained from *C. coli* strains RM1161 (A) and RM1051 (B), respectively. The relative intensities of the ions are shown on the *y* axis, and the masses (in Da) of the ions are shown on the *x* axis.

Similarly, a major ion of 10,031 Da (SIBI type 1), present in spectra for most *C. coli* strains (Fig. 2A), appeared at 10,061 Da (SIBI type 2) for at least one *C. coli* strain analyzed (Fig. 2B). Similar differences in ions were noted for *C. lari* strains at masses of 9,617 (SIBI type 1) and 9,650 (SIBI type 2) Da (Fig. 3A and B).

An ion at 13,729 Da was present in profiles for the majority of the *C. jejuni* reference strains and, thus, was designated a putative "common" ion (Table 2). However, this ion was missing in a few strains, and an ion of 12,887 or 12,888 Da was present instead (Table 2, RM1050, RM1158, and RM1159). It is not known whether these ions represent variant proteins or completely different proteins. However, subsequent analyses of two strains of *C. jejuni* subsp. *doylei* (RM2095 and RM2096)
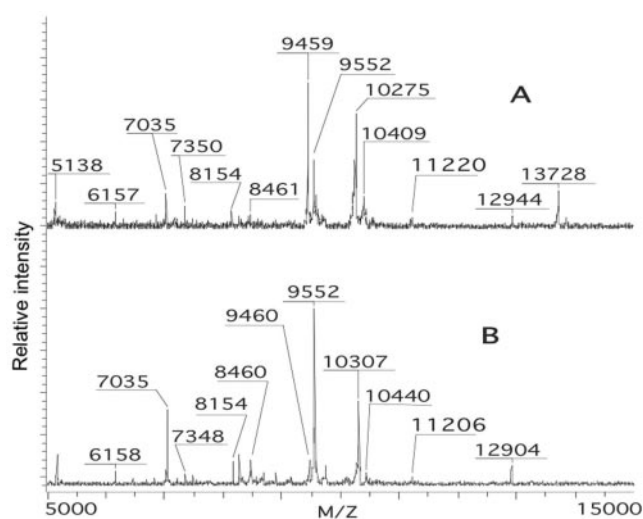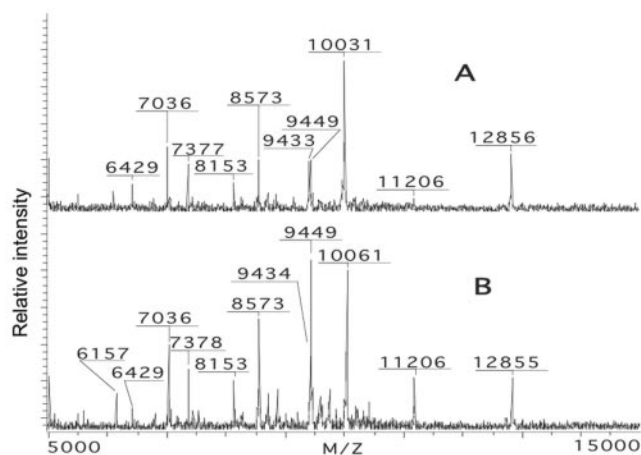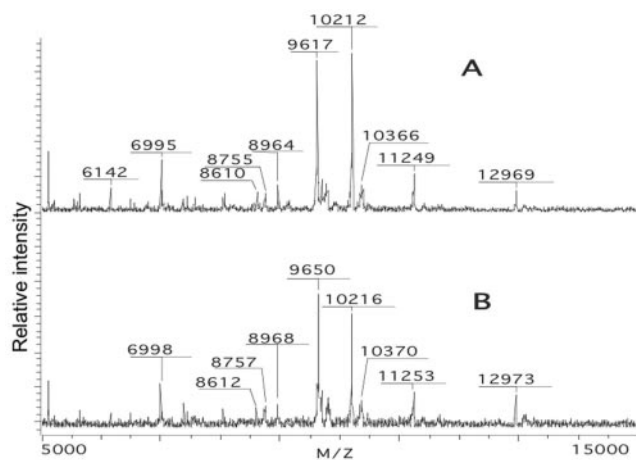

FIG. 3. MALDI-TOF MS spectra of whole-cell extracts of *C. lari* reference strains. Spectra representative of MS profile type 1 and profile type 2 were obtained from *C. lari* strains RM2100 (A) and RM2099 (B), respectively. The relative intensities of the ions are shown on the *y* axis, and the masses (in Da) of the ions are shown on the *x* axis.
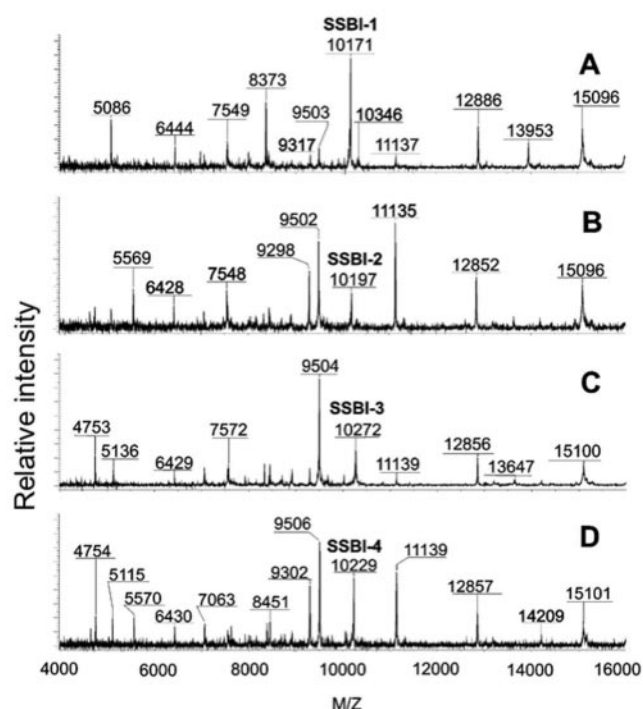
FIG. 4. MALDI-TOF MS spectra of whole-cell extracts of *C. upsaliensis* reference strains. Spectra for reference strains representative of SIBI types 1, 2, 3, and 4 were obtained with *C. upsaliensis* strains RM2092 (A), RM3776 (B), RM3195 (C), and RM4245 (D), respectively. The relative intensities of the ions are shown on the *y* axis, and the masses (in Da) of the ions are shown on the *x* axis. The SIBI types are designated above the singly charged ion for each strain.

identified the 10,276-Da *C. jejuni* type 1 SIBI, but they identified a 12,888-Da ion in the absence of the 13,729-Da *C. jejuni* common ion (data not shown). These results suggest that some of the *C. jejuni* reference strains (Table 2, RM1050, RM1158, and RM1159) and animal isolates (see Table S1 in the supplemental material [RM1216, RM1420, and RM1886]) may be *C. jejuni* subsp. *doylei*. Additional well-characterized *C. jejuni* subsp. *doylei* strains would be required to clarify this subspecies assignment.

MALDI-TOF analysis of *C. upsaliensis*. Four SIBIs differing by <60 Da in mass were identified for *C. upsaliensis* strains analyzed in this study. These *C. upsaliensis* SIBI types (designated 1 to 4) corresponded to ions at 10,171, 10,197, 10,272, and 10,229 Da, respectively (Fig. 4A to D). In addition, a common ion at 9,504 Da was observed for most of the *C. upsaliensis* reference strains (Table 2), regardless of SIBI type. Although this appeared to be a "common" *C. upsaliensis* SIBI, subsequent work indicated it was present also in *C. helveticus* strains (see below).

MALDI-TOF analysis of *C. helveticus*. Three reference strains of *C. helveticus*, including the type strain RM3228 (ATCC 51209), were analyzed by MALDI. An ion at approximately 10,189 Da was designated the *C. helveticus* SIBI-1 ion, since it was identified in all 10 *C. helveticus* strains analyzed (a 10,189-Da ion with a range of precision of ±10 Da gives a 10,199-Da ion as shown in Fig. 5A; this spectrum is an example of the ~10 Da difference between the mass calculated with external standardization and the actual mass). A common ion

TABLE 2. SIBIs identified for reference strains of *C. coli*, *C. jejuni*, *C. lari*, *C. sputorum* biovars and *C. upsaliensis* by MALDI-TOF MS[a]
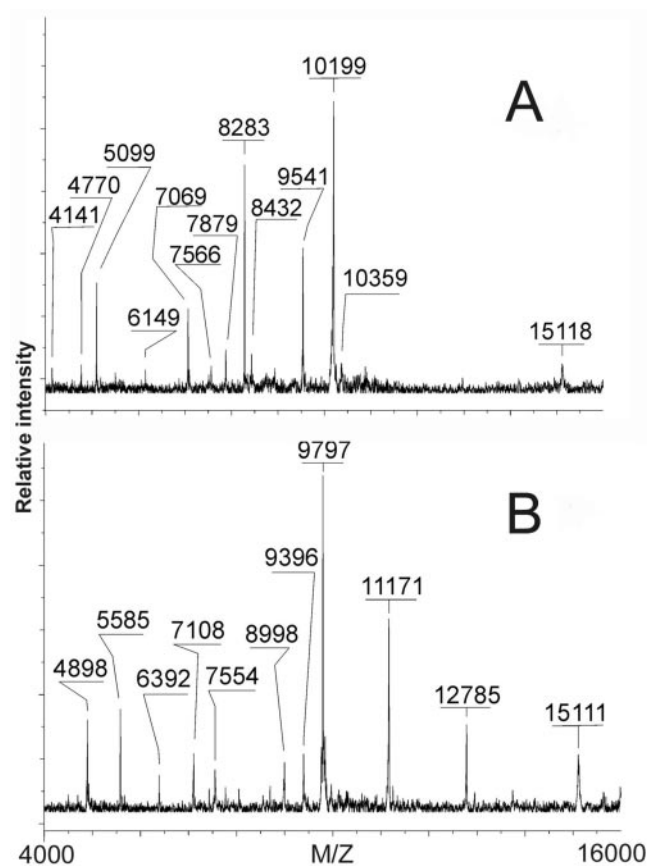
PSM strain no.	Species	Source[b]	Cc1 (10,032 Da)	Cc2 (10,060 Da)	Cc-C (12,855 Da)	Cj1 (10,276 Da)	Cj2 (10,303 Da)	Cj-C (13,729 Da)	Cl1 (9,619 Da)	Cl2 (9,651 Da)	Cl-C (12,972 Da)	Ch1 (10,189 Da)	Ch-C (9,504 Da)	Cu1 (10,171 Da)	Cu2 (10,197 Da)	Cu3 (10,272 Da)	Cu4 (10,229 Da)	Cu-C (9,504 Da)	Csp1 (9,797 Da)	Csp2 (9,817 Da)	Csp-C (12,786 Da)
RM1051	*C. coli*	H-CN	X		X																
RM1161	*C. coli*	H-CN	X	X[c]	X																
RM1166	*C. coli*	C-CN	X		X																
RM1169	*C. coli*	H-CN	X		X																
RM2228	*C. coli*	C-US	X		X																
RM2230	*C. coli*	C-US	X		X																
RM2231	*C. coli*	C-US	X		X																
RM2236	*C. coli*	C-US	X		X																
RM1048	*C. jejuni*	H-CN				X		X													
RM1050	*C. jejuni*	H-CN				X		VR[d]													
RM1155	*C. jejuni*	H-CN				X		X													
RM1156	*C. jejuni*	H-CN				X		X													
RM1862	*C. jejuni*	H-UK				X		X[d]													
RM1158	*C. jejuni*	H-CN				X		VR[d]													
RM1159	*C. jejuni*	H-CN				X		VR[d]													
RM1163	*C. jejuni*	H-CN				X		X													
RM1164	*C. jejuni*	H-CN				X		X													
RM1165	*C. jejuni*	C-CN				X	X[c]	X													
RM1167	*C. jejuni*	H-CN				X		X													
RM1168	*C. jejuni*	H-CN				X		X													
RM1462	*C. lari*	H-?							X												
RM2098	*C. lari*	H-US							X	X	X										
RM2099	*C. lari*	H-US								X	X										
RM2100	*C. lari*	H-US							X		X										
RM3228	*C. helveticus*	F-SW										X	X[e]								
RM4087	*C. helveticus*	F-SW										X	X								
RM4088	*C. helveticus*	F-S										X	X								
RM1485	*C. sputorum* bv. *fecalis*	O-?																	X		X
RM1495	*C. sputorum* bv. *bulbulus*	ND																	X		X
RM2089	*C. upsaliensis*	O-?												X							
RM2090	*C. sputorum* bv. *fecalis*	O-US																X	X		X
RM2091	*C. sputorum* bv. *fecalis*	O-?																	X		X
RM4120	*C. sputorum* bv. *paraureolyticus*	H-CN																		X	X
RM4121	*C. sputorum* bv. *fecalis*	O-UK																	X		X
RM1488	*C. upsaliensis*	H-US												X				X			
RM2092	*C. upsaliensis*	H-US												X				X			
RM2093	*C. upsaliensis*	H-US												X				X			
RM3937	*C. upsaliensis*	H-US												X				X			
RM3939	*C. upsaliensis*	H-US												X				X			
RM3940	*C. upsaliensis*	H-US												X				X			
RM3941	*C. upsaliensis*	H-US												X				X			
RM3942	*C. upsaliensis*	H-US												X				X			

Strain	Species	Source				
RM3943	C. upsaliensis	H-US	X			
RM3945	C. upsaliensis	D-US	X			
RM3946	C. upsaliensis	D-US	X			
RM3947	C. upsaliensis	D-US	X			
RM3948	C. upsaliensis	D-US	X			
RM3949	C. upsaliensis	D-US	X			
RM3950	C. upsaliensis	D-US	X			
RM4124	C. upsaliensis	D-SW	X			
RM4249	C. upsaliensis	H-BE	X			
RM4252	C. upsaliensis	H-BE	X			
RM3776	C. upsaliensis	H-SA		X		
RM3777	C. upsaliensis	H-SA		X		
RM4039	C. upsaliensis	H-SA		X		
RM3778	C. upsaliensis	H-SA		X		
RM4040	C. upsaliensis	H-SA		X		
RM4042	C. upsaliensis	H-SA		X		
RM4244	C. upsaliensis	H-BE		X		
RM4246	C. upsaliensis	H-BE		X		
RM4254	C. upsaliensis	H-BE		X		
RM3195	C. upsaliensis	H-SA			X	
RM4245	C. upsaliensis	H-BE				X
RM4248	C. upsaliensis	H-BE				X
RM4258	C. upsaliensis	H-BE				X
RM4250	C. upsaliensis	H-BE				X
RM4257	C. upsaliensis	H-BE				X
RM4258	C. upsaliensis	H-BE				X

[a] SIBIs identified for each strain are designated with an X. Multiple SIBIs were identified for most species. SIBI type 1 (e.g., Cc1) refers to the biomarker ion observed consistently among the strains tested of the designated species and the ion mass; SIBI type 2 (e.g., Cc2) designates a second high-intensity SIBI. The same designations apply for the other SIBIs.
[b] Abbreviations: H, human; C, chicken; F, feline; O, ovine; D, canine; CN, Canada; US, United States; SW, Switzerland; S, Sweden; BE, Belgium; SA, South Africa; UK, United Kingdom; ND, not determined.
[c] Two biomarker ions were present for this "strain."
[d] VR, variant result. No C. jejuni common ion was identified; a 12,887- or 12,888-Da ion was noted.
[e] Ch-C ion for RM3228 (C. helveticus type strain) was 9,532 Da, ~28 Da higher than the Ch-C observed for other C. helveticus strains.

FIG. 5. MALDI-TOF MS spectra of whole-cell extracts of *C. helveticus* and *C. sputorum* reference strains. Spectra for *C. helveticus* reference strain RM3228 (A) and *C. sputorum* reference strain RM1485 (B) are shown. The relative intensities of the ions are shown on the *y* axis, and the masses (in Da) of the ions are shown on the *x* axis.
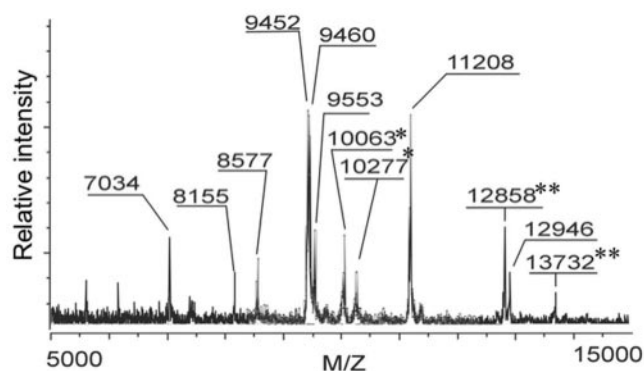

FIG. 6. MALDI-TOF MS spectra of a whole-cell extract of *C. coli* and *C. jejuni* cells in a sample expected to be a pure culture of either *C. coli* or *C. jejuni*. For strain RM1882, both *C. coli* and *C. jejuni* SIBIs (*) and common ions (**) were evident in the spectrum (also see Table S1 in the supplemental material).

at 9,504 Da was noted for two of the three reference strains (RM4087 and RM4088) but was 28 Da lower than an ion at 9,532 Da observed consistently for the type strain of *C. helveticus*, RM3228 (9,541 Da − 10 Da = 9,531 Da [Fig. 5A]). Nevertheless, the *C. helveticus* common ion was designated 9,504 Da, since subsequent studies with additional *C. helveticus* strains revealed that this was the major *C. helveticus* common ion observed (see Table S1 in the supplemental material). As noted above, the Ch-C ion at 9,504 Da was also a common ion for *C. upsaliensis* SIBI types 1 to 4. Thus, observance of an ion at 9,504 Da indicated a putative *C. helveticus* or *C. upsaliensis* strain, necessitating accurate analysis of the *C. helveticus* SIBI at 10,189 Da and the type 2 *C. upsaliensis* SIBI at 10,197 Da. It is worth noting that multiple common ions at masses of <9,000 Da also were observed for all 10 *C. helveticus* strains analyzed (e.g., 5,094 ± 2 and 8,451 ± 8 Da), also facilitating species determination.

MALDI-TOF analysis of *C. sputorum*. Four reference strains of *C. sputorum* biovar *fecalis* isolated from sheep and a single strain each of *C. sputorum* biovars *bulbulus* and *paraureolyticus* were analyzed by MALDI-TOF MS. A profile of the *C. sputorum* biovar *bulbulus* reference strain RM1495 is shown in Fig. 5B. Possible SIBIs and/or common ions at 9,796, 11,170, and 12,786 Da were noted in five of six *C. sputorum* reference

strains (Table 2). The 9,796-Da ion was designated SIBI type 1 because it was present in spectra for each of the four *C. sputorum* biovar *fecalis* strains and the single *C. sputorum* biovar *bulbulus* strain, and it generally was of high intensity. A comparable ion at 9,817 Da (~20-Da difference) was present for the single *C. sputorum* biovar *paraureolyticus* strain (RM4120). Similarly an ion at 11,203 Da was present for *C. sputorum* biovar *paraureolyticus* instead of the 11,170-Da ion (~30-Da difference). Both of these ions may be useful as *C. sputorum* type 1 SIBIs.

Strain RM2089 was received for analysis as a *C. sputorum* biovar *fecalis* strain, but our analysis determined it to be *C. upsaliensis* based on a prominent *C. upsaliensis* SIBI at 10,181 Da and a *C. upsaliensis*/*C. helveticus* common ion at 9,509 Da. This strain was confirmed subsequently as *C. upsaliensis* in an expanded MLST system developed for genotyping *Campylobacter* (52).

Additional ions that appeared to be potential SIBIs were observed for each of the six species, e.g., 9,459 (or 9,460) Da for *C. jejuni*, 8,573 Da for *C. coli*, 10,212 (to 10,216) Da for *C. lari*, 11,135 to 11,139 Da for *C. upsaliensis*; 8,452 to 8,460 and 15,100 to 15,130 for *C. helveticus*, and 11,170 Da for *C. sputorum* (Fig. 1 to 5). These ions were not selected as prototype SIBIs because they were not as clearly a species-identifying ion, were of lesser abundance, and/or were not as conserved in a species as the ions selected as SIBIs.

MALDI-TOF analysis of putative or suspected *C. coli* and *C. jejuni* strains. More than 50 strains suspected of being *C. coli* or *C. jejuni*, plus recent isolates from environmental sources (including poultry and swine carcasses and dog and cat feces), were analyzed by MALDI-TOF MS, and the results were analyzed to determine species (see Table S1 in the supplemental material). For the *C. coli* and *C. jejuni* strains, the MS results were compared also to results obtained with anti-*C. coli* and anti-*C. jejuni* specific MAbs and a hippuricase biochemical assay (see Table S1). Either a *C. coli* or a *C. jejuni* SIBI was identified for all suspected *C. coli* or *C. jejuni* strains, resulting in unambiguous identification of the species.

Identification of mixed *C. coli* and *C. jejuni* "strains." For most experiments, data were consistent with the immuno-chemical and biochemical results (see Table S1 in the supplemental material). However, inconsistent results were observed
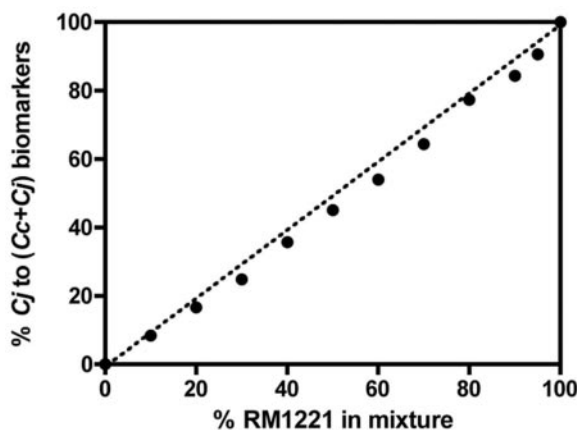
FIG. 7. Detection of a *C. jejuni* biomarker ion in a mixture of *C. jejuni* strain RM1221 and *C. coli* strain RM2228 whole cells. *C. coli* and *C. jejuni* cells were suspended in different proportions ranging from 0 to 100% of the total. The results are plotted as the percentage of the intensity of the *C. jejuni* biomarker ion measured by MS compared to the percentage of *C. jejuni* in the mixture.

in the spectrum for "*C. jejuni*" strain RM1882 (Fig. 6). Close examination of the profile revealed that both a *C. coli* and a *C. jejuni* SIBI were present in the sample assumed to be a pure culture of *C. jejuni* (Fig. 6). Eight single-colony picks were obtained for "*C. jejuni*" RM1882 and tested separately for hippuricase activity. Six of the eight single-colony picks were positive for hippuricase activity, suggesting that "strain" RM1882 was a mixture of *C. jejuni* and *C. coli* or that hippuricase-negative *C. jejuni* was present. The hippuricase-positive and hippuricase-negative isolates were analyzed, and the immunochemical and MALDI-TOF MS results were consistent with the presence of both *C. coli* and *C. jejuni* colonies: the isolated strains expressed either a *C. coli* or a *C. jejuni* SIBI, but not both (data not shown).

MALDI-TOF MS of *C. lari* strains. Four strains received as either *C. coli* or *C. jejuni* (RM1887 to RM1890) (Table 1; see also Table S1 in the supplemental material) were of particular interest because they lacked the characteristic *C. coli* and *C. jejuni* SIBI. The spectrum for strain RM1889 contained prominent ions at 6,995, 9,648, 10,212, 11,250, and 12,970 Da (see spectra in Fig. 3 for other *C. lari*). In subsequent studies, all four strains were confirmed as predominately *C. lari* by comparison with MS data obtained with *C. lari* reference strains (Fig. 3). A minor *C. jejuni* SIBI (10,278-Da) peak was observed in the spectra for *C. lari* RM1890 (see Table S1). No attempt was made to isolate a possible *C. jejuni* from this sample, since MLST results did not reveal evidence of any other species contaminating RM1890 (data not shown).

MALDI-TOF MS of *C. helveticus* and *C. upsaliensis* strains from cats and dogs. Thirteen strains of suspected *C. helveticus* or *C. upsaliensis* isolated in our laboratory from dog and cat feces by a membrane filtration method were analyzed with MALDI-TOF MS. Initial results suggested that at least six of the strains had a prominent type 1 *C. upsaliensis* SIBI (10,171 Da); the remaining seven strains had a prominent ion ranging from 10,188 to 10,194 Da, a mass closest to the type 2 *C. upsaliensis* SIBI (10,197 Da). In addition, the *C. upsaliensis* common ion was identified for 12 of 13 strains (see Table S1 in

the supplemental material). A 16S rRNA analysis indicated that seven strains with the 10,188- to 10,194-Da ions were either *C. upsaliensis* or *C. helveticus*, but the exact species could not be determined based on comparisons to *C. helveticus* and *C. upsaliensis* 16S data in GenBank (4). However, analyses of these strains by an MLST method developed by our laboratory confirmed them as *C. helveticus* (see Table S1) (52). The common ion identified for *C. upsaliensis* strains (9,504 Da) was expressed also by *C. helveticus* strains and, fortuitously, was not observed in profiles for other *Campylobacter* species. Therefore, the 9,504-Da ion (*C. upsaliensis*/*C. helveticus*) and accurate determination of the *C. upsaliensis* and *C. helveticus* SIBIs (±1 Da with internal standard calibration) allowed differentiation of these two closely related species. All of the *C. helveticus* strains in this set were isolated from cats; three *C. upsaliensis* strains each were isolated from cats and dogs (Table 1; see also Table S1). A *C. upsaliensis* type 1 SIBI (10,171 Da) was present also in profiles of multiple analyses of putative *C. helveticus* strain RM3807, indicating either that it was composed of a mixture of *C. upsaliensis* and *C. helveticus* cells or that both types of SIBIs can be expressed by this strain (e.g., a chimera). Analyses of additional *C. helveticus* strains will be required to determine whether this *C. upsaliensis*-specific SIBI can occur in other *C. helveticus* strains.

Detection sensitivity of *C. coli* and *C. jejuni* in a mixture. To determine the detection sensitivity of *C. coli* or *C. jejuni* in mixed cultures under conditions similar to those encountered for analyses of unknown strains, *C. jejuni* strain RM1221 and *C. coli* strain RM2228 were analyzed as a mixture at different proportions of each strain. Cells were removed from agar, combined in proportions varying from 10 to 100% for each strain, and then tested by MALDI-TOF MS. The results shown in Fig. 7 illustrate that the intensity of the *C. jejuni* and *C. coli* SIBI (at 10,277 Da [*C. jejuni* SIBI type 1] and 10,063 Da [*C. coli* SIBI type 2]) corresponded directly to the concentration of each strain. These results suggest that, at least under these ideal experimental conditions, different species of *Campylobacter* with SIBIs distinguishable by MALDI-TOF MS can be detected when the strain in lowest concentration is at least 5 to 10% of the mixture.

It is worth noting that a single sample of bacteria harvested from agar plates likely will not reflect exactly the overall proportion of different species and strains in a mixed culture due to heterogeneity in the distribution on a plate. Different *Campylobacter* species and strains mixed in complex environmental samples can be difficult, if not impossible, to distinguish on selective or nonselective media. To illustrate the potential colony morphology encountered on plates sampled for analysis, a *C. jejuni* strain and a *C. lari* strain expressing CFP (*C. jejuni*-CFP) or GFP (*C. lari*-GFP), respectively, were mixed in different proportions (1:1 *C. lari*-GFP/*C. jejuni*-CFP [Fig. 8B]; 1:10 *C. lari*-GFP/*C. jejuni*-CFP [Fig. 8C]), plated, and incubated, and then growth was examined under a fluorescence stereomicroscope. *C. jejuni* (Fig. 8) often grew as large and heterogeneously shaped colonies that result from swarming motility. In contrast, *C. lari* growth was present predominantly in small, round colonies (Fig. 8A to C). *C. lari*-GFP colonies were observed often surrounded, but not covered, by confluent *C. jejuni*-CFP growth, or on top of confluent *C. jejuni* growth. Colonies of *C. lari* and *C. jejuni* growing next to one
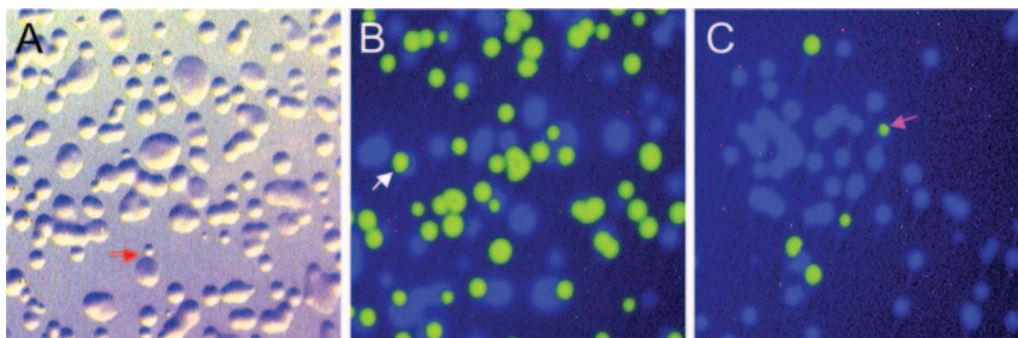
FIG. 8. Mixed culture of *C. jejuni*-CFP and *C. lari*-GFP on BAB medium. *C. jejuni*-CFP and *C. lari*-GFP cells were harvested from BAB medium and suspended in broth in proportions of 1:1 (B) or 10:1 (C), respectively, mixed, and then plated on BAB. The plates were incubated for 30 h, and growth was observed under a fluorescence stereomicroscope (Leica MZIII). (A) No filters; the image shows growth of a 1:1 proportion of *C. jejuni* and *C. lari*. Note the larger colonies of *C. jejuni*-CFP (blue) compared to *C. lari*-GFP (green) (B and C). The red arrow points to a *C. lari* colony next to a *C. jejuni* colony (A); the white arrow in panel B points to a *C. lari*-GFP colony on top of a *C. jejuni*-CFP colony; the violet arrow in panel C points to a small *C. lari*-GFP colony on top of *C. jejuni*-CFP growth.

another and indistinguishable under regular light were sometimes observed (Fig. 8C, top). No sectored colonies corresponding to a mixed strain "CFU" were observed in this experiment. These results reflect the importance of proper sampling and analysis of multiple samples to obtain a thorough assessment of the diversity of *Campylobacter* strains and species that may be present in either clinical or environmental samples.

Consistency of spectra of *C. jejuni* strain RM1221 after growth on different media and under different atmospheric conditions. To determine whether the SIBI expression was stable under different culture conditions, *C. jejuni* strain RM1221 was subcultured on three different media under two atmospheric conditions at two different temperatures, and samples of bacteria were analyzed by MALDI-TOF MS. The masses determined for the *C. jejuni* SIBI type 1 (10,276 Da), the *C. jejuni* common ion (13,729 Da), and a third ion at ~9,551 Da (probable 30S ribosomal protein S17) were very reproducible for samples of *C. jejuni* RM1221 grown under each of nine combinations of conditions (see Table S2 in the supplemental material). No significant differences were noted in the mass of high-intensity ions, regardless of the media (e.g., blood), gas mixture (5% or 10% CO_2 and 5% H_2 or 5% O_2), or incubation temperature (37°C versus 42°C), as shown in the following results: *C. jejuni* SIBI type 1 = 10,276.0 ± 1.3; *C. jejuni* common SIBI = 13,729.0 ± 1.6; "ion 2" = 9,551.0 ± 1.4. In addition, eight replicate samples from a plate of *C. coli* RM2228, *C. lari* RM2100, and *C. upsaliensis* RM3195 grown under a single condition (BAB medium, 10% CO_2–5% H_2, 37°C) were analyzed (see Table S2). The standard deviations for the replicates for the three ions for the three strains ranged from 0.9 (*C. coli* SIBI type 1) to 3.1 (*C. upsaliensis* ion 2), reflecting good intraexperimental reproducibility. A similar experiment with three different strains of *C. helveticus* (RM3228, RM4087, and RM4088) grown under the same conditions resulted in average values of 10,188.3 ± 1.4, 9,522.1, and 9,504.0 ± 0.7 for the *C. helveticus* SIBI, *C. helveticus* common SIBI for RM4087 and 4088, and *C. helveticus* common SIBI for RM3228, respectively, for six samples total (two for each strain) for each ion (data not shown). Two strains of *C. sputorum* biovar *fecalis* (RM1495 and RM4121) and *C. sputorum*

biovar *paraureolyticus* strain RM4120 were analyzed in a second experiment with replicate samples. The average values for the SIBI and common SIBI for the two *C. sputorum* biovar *fecalis* strains were 9,797.1 ± 1.26 and 11,167 ± 2.61, respectively; for the single *C. sputorum* biovar *paraureolyticus* strain the values were 9,813.7 ± 0.1 and 11,193 ± 0.4 (data not shown). These values reflect the potential difference between *C. sputorum* biovar *fecalis*/*C. sputorum* biovar *bulbulus* and *C. sputorum* biovar *paraureolyticus* described above (Table 2), although additional strains of each biovar would need to be tested to confirm this biovar-specific difference.

These results indicate that conventional growth conditions do not influence markedly the mass of ions detected. It is worth noting, however, that horse blood-related proteins apparently incorporated by the cells or into CFU from the underlying blood agar medium were identified in some profiles. This was confirmed in control experiments with samples of BAB medium extracted directly from plates without bacteria (e.g., 14,890, 15,050, 15,100, and 16,075 Da). We suspected that the 16,075-Da ion corresponded to the β-chain protein of horse hemoglobin (61). This was exploited in later experiments as a useful internal protein standard of an appropriate mass for calibration, thus facilitating extremely accurate mass assignments of SIBIs (±1 Da) for identification of *C. helveticus*, *C. upsaliensis*, and *C. sputorum* biovar *fecalis* strains (see Table S1 in the supplemental material).

DISCUSSION

We have presented data that multiple *Campylobacter* species grown on solid medium under a variety of conditions can be analyzed by MALDI-TOF MS to yield high-intensity and, in most cases, intact protein ions in the 9- to 14-kDa range that are diagnostic of *Campylobacter* species. Pure cultures of *Campylobacter* can be analyzed rapidly with less ambiguous results, compared to many methods used currently for confirming species. An added advantage of MALDI-TOF MS is that multiple species of *Campylobacter* in mixed cultures can be identified more easily by MS than by conventional methods; reproducibility of the MALDI-TOF MS protein profiles for

TABLE 3. Summary of SIBIs for six *Campylobacter* species

Species	SIBI type	Observed mass (Da)[a]	Confirmed, or possible, protein identity[b]	Predicted mass (Da)
C. coli	Common	12,855	50S ribosomal, L7/L12 (possible), posttranslationally modified[c]	12,985
C. coli	1	10,032	DNA binding, HU homolog	10,030
C. coli	2	10,060	DNA binding, HU homolog	10,060[j]
C. jejuni	Common	13,729	30S ribosomal, S13 subunit	13,735
C. jejuni	1	**10,276**[d]	DNA binding, HU homolog	10,274
C. jejuni	2	10,303	DNA binding, HU homolog[e]	10,304[j]
C. lari	Common	12,972	50S ribosomal, L7/L12 or L18 (possible), posttranslationally modified[f]	13,099/13,100
C. lari	1	9,619	GroES chaperonin (cpn10)	9,617
C. lari	2	9,651	Conserved hypothetical	9,655
C. sputorum biovars	Common	12,786	Not determined	Unknown
C. sputorum biovars	1	9,796[g]	Not determined	Unknown
C. helveticus	Common	**9,504**	Not determined	Unknown
C. helveticus	1	**10,189**[h]	DNA binding, HU homolog	10,187[j]
C. upsaliensis	Common	**9,504**	Conserved hypothetical	9,503
C. upsaliensis	1	10,171	DNA binding, HU homolog	10,172[j]
C. upsaliensis	2	**10,197**	DNA binding, HU homolog	10,199[j]
C. upsaliensis	3	**10,272**[i]	DNA binding, HU homolog	10,271
C. upsaliensis	4	10,229	DNA binding, HU homolog	10,227[j]

[a] Masses shown in boldface type denote ions of <10 Da different from a SIBI for another species: *C. helveticus* common and *C. upsaliensis* common (0 Da), *C. helveticus* type 1 and *C. upsaliensis* type 2 (8 Da), *C. jejuni* type 1 and *C. upsaliensis* type 3 (4 Da).

[b] The potential protein was determined from the predicted protein masses for genes annotated for the completed genomes of *C. jejuni* strains NCTC 11168 (see Parkhill et al. [58]) and partial genome sequences of *C. coli* RM2228 (*C. coli* SIBI type 1), *C. lari* RM2100 (*C. lari* SIBI type 1), and *C. upsaliensis* RM3195 (*C. upsaliensis* SIBI type 3) (65).

[c] A predicted protein mass was present in the *Cc* RM2228 database at 12,851 Da. However, the best candidate protein for this *Cc* SIBI is a ribosomal protein minus the N-terminal methionine (12,985 Da minus 131 Da = 12,854 Da).

[d] The putative gene encoding this protein is designated *Cj0913c* for *C. jejuni* strain NCTC 11168. The identity of this protein was confirmed by tryptic mass mapping analysis (data not shown).

[e] A cytochrome *c* oxidase protein (cbb3 type, subunit IV) of predicted mass 10,303 Da is annotated in *C. jejuni* RM1221; however, a high-copy-number HU protein with one or more single amino acid changes is a more probable candidate for the type 2 *C. jejuni* SIBI.

[f] No predicted protein mass was present in the *C. lari* RM2100 database within ±10 Da of 12,972. Two candidates for this *C. lari* SIBI are two *C. lari* ribosomal proteins with the loss of the N-terminal methionine (13,099 or 13,100 Da minus 131 Da = 12,968/12,969 Da).

[g] The ion nearest in mass for a single strain of *C. sputorum* bv. *paraureolyticus* (RM4120) was 9,817 Da.

[h] The *C. helveticus* strains were presumed to be *C. upsaliensis* strains (see Table S1 in the supplemental material). The predicted mass for this SIBI type is an actual mass for strain RM3807 determined by tryptic mass mapping analysis (18).

[i] The identity of this protein was confirmed by tryptic mass mapping analysis.

[j] Determined by *hup* sequencing (18).

C. coli and *C. jejuni* strains was sufficient to allow species identification of isolates.

Summary of *Campylobacter* SIBI types. The different types of SIBIs, their approximate masses, and the candidate proteins identified for each ion for six species of *Campylobacter* are summarized in Table 3. A comparison of the SIBI masses illustrates that in most cases there are sufficient differences between SIBIs (>12 to 15 Da) for accurate identification of isolates and mixed species cultures by MALDI-TOF MS. However, there are three sets of SIBIs that are either identical (*C. helveticus* and *C. upsaliensis* common ions) or very similar (<10-Da difference for *C. helveticus* type 1 and *C. upsaliensis* type 2 and for *C. jejuni* type 1 and *C. upsaliensis* type 3) that require very accurate mass assignments. The level of accuracy required to differentiate these species was achieved by internal calibration with standard proteins or blood proteins from the medium.

The identification of *Campylobacter* species based on the presence or absence of biomarker proteins in the selected mass range from 9 to 14 kDa was 100% accurate for the 76 samples analyzed in our semiblinded study (see Table S1 in the supplemental material). Generally, the species of the strain could be determined by the presence of a single SIBI (e.g., Table 2, SIBI types). However, additional slightly higher mass ions were

usually present to assist in confirming the species (e.g., "common" ions).

The approach to species identification for *Campylobacter* strains presented in this study is based on the presence or absence of SIBIs. However, we have noted variation in the SIBI masses for certain strains, arising from possibly nonsynonymous mutations in genes encoding the SIBIs and resulting in protein mass shifts that allow some limited differentiation of the strains. For example, a 10,276-Da ion was present in 13 of 14 strains (93%) of the reference strain set of *C. jejuni* (Table 2) used to identify potential SIBIs (a 10,303-Da ion occurred in one strain, *C. jejuni* RM1165). However, 6 of 31 (19%) putative *C. jejuni* strains tested in the semiblinded samples (see Table S1 in the supplemental material) expressed the ~10,303-Da ion (SIBI type 2). Six of the seven SIBI type 2 *C. jejuni* strains were isolated from chickens (Canada, Texas, and California), possibly indicating an association of SIBI type with source.

Table 2 and also Table S1 in the supplemental material include only a subset of the ions detected in both *C. coli* and *C. jejuni* strains. Although the *C. jejuni* 13,729-Da ion occurred at approximately the same mass in most of the *C. jejuni* strains, it was absent in six human and animal *C. jejuni* strains and was replaced by an ~12,884- to 12,888-Da ion (~30 Da higher than

the *C. coli* common ion) (Table 2; see also Table S1 in the supplemental material). The "12,888 ion" was identified also in the only *C. jejuni* subsp. *doylei* strains we had available for analysis (both human clinical strains). Similarly, the *C. coli* SIBI at 12,857 Da was absent in a few strains tested, and an ion at 12,914 Da was observed (57-Da difference). These results indicate the importance of identifying multiple SIBIs for accurate analysis and identification and the potential value of MALDI-TOF analysis for identifying subspecies/biovar differences in *Campylobacter*.

In a previous study of a few *C. coli* and *C. jejuni* strains by MALDI-TOF MS, biomarker ions were identified at 10,074 Da for *C. coli* strain ATCC 43474 and at 10,285 Da for *C. jejuni* strain ATCC 43464 (73). It is probable that these ions correspond to the *C. coli* type 2 (10,060 Da) and *C. jejuni* type 1 (10,276 Da) SIBIs identified in our study. The 9- and 14-Da mass differences for these ions in the two studies may reflect an actual difference (e.g., amino acid polymorphism or posttranslational modification) or differences in measurement precision. However, our analysis of the same *C. coli* strain as the previous study (RM1878) (Table 1; see also Table S1 in the supplemental material) suggests the difference is due to precision.

Identities or putative identities of some SIBIs. Many of the SIBIs identified in this study are putatively abundant, cytosolic proteins, of relatively low mass compared to the majority of proteins, and apparently conducive to ionization in MALDI-TOF MS (Table 3, HU-homolog, ribosomal subunit, and chaperonin proteins). A *C. jejuni* HU homolog encoded by the *hup* gene was characterized previously by Konkel et al. and designated HCj (33). The calculated molecular mass of HCj was 10,267 Da, with a pI of 10.1. Presumably, this small, highly basic, histone-like protein has properties similar to other HU proteins with regards to abundance (30,000 to 50,000 HU dimers per cell in *E. coli*) and DNA binding and condensing activity (14). It is probable that the high intensity of HU/HCj and possible HCj-homologs in other *Campylobacter* species is due to a combination of similar high abundance plus efficient ionization of these highly basic proteins. In addition, we speculate that cytosolic proteins whose functions and sequences have been highly conserved through evolution are good candidates for species identification, although of limited utility for strain differentiation. It will be interesting to determine whether these same high-intensity ions identified by MALDI-TOF MS (e.g., the HU protein) are good predictors of other *Campylobacter* species (18) and bacterial species of other genera.

The possible identities for some of the SIBIs shown in Table 3 must be considered speculative until confirmed by tryptic mass mapping experiments. For example, the *C. coli* and *C. lari* common SIBIs have been designated as 50S ribosomal L7/L12 proteins (RplL), even though the predicted protein is ~130 Da larger than that observed by MALDI-TOF MS. This is because no predicted protein mass closer than 10 Da was present in the genome database, suggesting that post- or cotranslational modification had occurred, resulting in removal of the N-terminal methionine (131 Da) by a specific aminopeptidase (13, 27).

Polymorphisms and variation of SIBIs. Ongoing genetic and mass mapping studies in our laboratory indicate that the mass differences between SIBI types, in fact, are due to nonsynonymous single or multiple nucleotide polymorphisms, rather than

differences due to sensitivities of methods (18). These SIBI types representing authentic nonsynonymous mutations will be useful for population analyses of strains. Strains related epidemiologically (e.g., from an outbreak or prevalent in a single source) can be identified based on a pattern of small mass differences reflecting single or multiple amino acid changes that are different from other strains. For example, all of the *C. upsaliensis* strains from the United States were *C. upsaliensis* SIBI type 1 (~10,171 Da), in contrast to six of seven *C. upsaliensis* strains from South Africa, which were *C. upsaliensis* SIBI type 2 (~10,197 Da). However, of the 11 *C. upsaliensis* reference strains from Belgium, two, three, and six strains represented SIBI types 1, 2, and 4, respectively. The common ion at 9,504 Da was observed in all of the *C. upsaliensis* MALDI-TOF MS profiles. The four *C. upsaliensis* SIBI types are consistent with the genomic heterogeneity reported previously for animal and human (8, 40, 57) and, specifically, dog and cat *C. upsaliensis* strains (40, 54). Twelve of 13 dog and cat *C. upsaliensis* strains in our study were from the United States, and all were *C. upsaliensis* SIBI type 1. Although SIBI types provide probably only a minor discrimination of strains, it will be interesting eventually to compare SIBI types with sequence types determined by a new MLST method for *C. coli*, *C. lari*, *C. helveticus*, and *C. upsaliensis* (52).

As we expand our MALDI-TOF MS analyses to include other species of *Campylobacter*, it is anticipated that protein homologs to the SIBIs described in this study will be identified as biomarkers. For example, we have identified recently a 10.5-kDa protein as the HU protein, and a potential SIBI, for *C. concisus* strains (18). The probability will increase that SIBIs in different species with similar masses (~10 Da) will be identified, again emphasizing the need for accurate mass assignments. As noted above, the identification of medium-derived horse hemoglobin β-chain in some experiments permitted recalibration of data, yielding ±1 Da accuracy that proved to be essential for differentiating the *C. helveticus* SIBI (~10,189 Da) from a *C. upsaliensis* type 2 SIBI (~10,197 Da). Thus, media proteins distinct from bacterial proteins, or addition of a reference protein to each sample, can be exploited for internal calibration to improve precision and accuracy of mass measurement.

The correct species designations for >130 strains analyzed in this study were identified usually by identifying only two SIBIs: a single SIBI among multiple variant SIBIs (e.g., *C. upsaliensis*), plus a SIBI common among all or most strains of a species. However, it is worth emphasizing that additional SIBIs are observed in the mass range of 7 to 15 kDa that permit confirmation of species when SIBIs for different species are of similar mass (±10 Da) or are variable among environmental strains of the same species. Therefore, identifying a set of SIBIs would be advantageous for unambiguous assignments of species and important intraspecies strain differences.

As more organisms are sequenced and the data are entered into public databases (e.g., NCBI GenBank), the accuracy in identification and analysis of unknown isolates by proteomics approaches also will increase (12, 13, 19). In a single study reporting species identification by querying ion masses against protein databases (19), organisms could be identified only to the genus level. In our study, and other studies, isolates are classified tentatively as members of a particular genus and/or

species through both sample source and the special selection and enrichment conditions involved in obtaining them. MALDI-TOF MS has proved to be useful for rapid confirmation of species.

Environmental samples and mixed species cultures. Selective media for isolation of *C. coli* and *C. jejuni* from clinical or environmental (animals, food, and water) samples commonly include antibiotics for minimizing other microbial flora. The unintended consequence of this approach is that antibiotic-sensitive strains of *C. coli* and *C. jejuni* and other *Campylobacter* species are not culturable. MALDI-TOF MS speciation is very efficient with pure cultures of *Campylobacter*. However, we have shown also that multiple *Campylobacter* species can be identified with a preparation of *Campylobacter* cells growing confluently, or as single colonies, on common agar media (Fig. 8). We are developing an approach currently to analyze multiple colonies of suspected *Campylobacter* species obtained following passage through 0.6-μm filters and growth on nonselective culture medium in the appropriate atmosphere (22, 39). Since multiple strains of a majority of *Campylobacter*, *Helicobacter*, and other spiral-form bacteria could be present and difficult to distinguish, MALDI-TOF MS analysis provides a simple method for identifying potential *Campylobacter* species, and also potential mixed-species colonies (Fig. 8), as has been reported previously (17, 50). We confirmed numerous times during this study that "isolates" received from other sources were either speciated incorrectly or contained multiple species.

Intraspecies variability of the *Campylobacter* SIBIs, potentially due to single or multiple amino acid sequence changes, has been identified; these data are not attainable rapidly in most other assays. MALDI-TOF MS may be especially useful for characterizing non-*C. coli* and non-*C. jejuni* strains because of the minimal genetic and biochemical data available for emerging *Campylobacter* species (10). The recent increase in genomic sequence data available for *Campylobacter* species will enhance MALDI-TOF MS as a method of analysis for *Campylobacter* (21, 65), since accurate protein masses yield gene and protein identifications for strain differentiation and further analysis.

ADDENDUM IN PROOF

We have recently created a database of all of the MALDI-TOF MS data acquired in our laboratory for *Campylobacter* species and other species of bacteria, as well as a program (M2MASS) for comparing all of the ions detected for a strain with ions obtained for every other strain represented in the database. This analysis yields a score for determining the similarities between mass data among strains and assigning species. Within the program, the masses of selected ions can be compared to predicted masses for genes in public and PSMRU sequence databases, facilitating comparisons of large amounts of data and, thus, increasing the accuracy of the conclusions.

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